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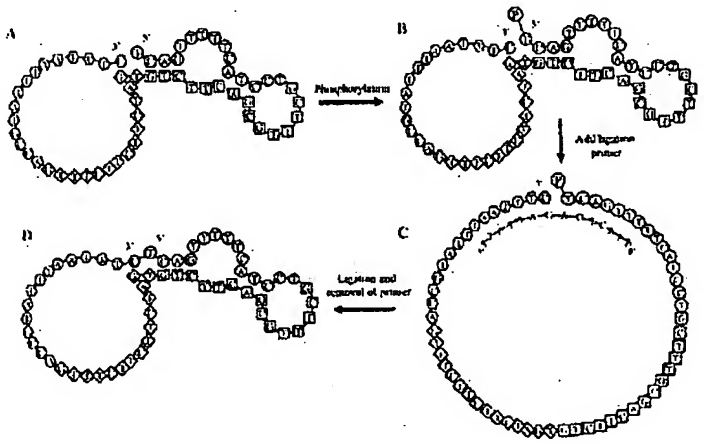

(57) Abstract

The present invention relates to novel antisense (AS) oligos containing one or more antisense sequence to mRNA region with a less secondary structure. Particularly, the present invention relates to a covalently-closed multiple antisense (CMAS)-oligo, which is constructed to form a closed type by ligation using complementary primer, and a ribbon-type antisense (RiAS)-oligo, which is composed of two loops containing multiple antisense sequences and a stem connecting the two loops that is constructed to by ligation using complementary sequences at both 5 prime ends. Since the novel AS-oligos of this invention are extremely stable to exonuclease activities, and show a significant growth inhibition of tumor cells, pharmaceutical compositions containing the novel types of AS-oligos of the invention are effective for treatment cancer, immune diseases, infectious diseases, and other human diseases caused by aberrant gene expression.

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THE NOVEL ANTISENSE-OLIGOS WITH BETTER STABILITY AND ANTISENSE EFFECT

FIELD OF THE INVENTION

The present invention relates to novel antisense oligos containing one or more antisense sequence to mRNA region with a less secondary structure to improve their target sequence specificity and stability to nuclease activities.

Particulary, the present invention relates to covalently-closed multiple antisense(CMAS)-oligos containing multiple target antisense sequences to various protooncogene mRNAs including c-myb, c-myc, or k-ras. The CMAS-oligos are constructed to form a closed type by ligation using complementary primers.

In addition, the present invention relates to ribbon-type antisnese(RiAS)-oligos containing multiple target antisense sequences to various protooncogene mRNAs including c-myb, c-myc, or k-ras. The RiAS-oligos are constructed to form a stem-loop structure by ligation using complementary sequences at both 5 prime ends.

The present invention relates to pharmaceutical

composition containing the novel types of AS-oligos for treatment cancer, immune diseases, infectious diseases, and other human diseases caused by aberrant gene expression.

BACKGROUND

Antisense oligonucleotides(hereinafter, referred to as 'AS-oligos') have been valuable in the functional study of a gene by reducing expression of the gene in a sequence specific manner(Thompson, C. B. et al., Nature, 314, 363-366, 1985). Intense efforts have also been made to develop molecular antisense agents by ablating aberrant expression of genes involved in tumor initiation and progression(Chavany, C. et al., Mol. Pharm., 48, 738-746, 1995).

Synthetic AS-oligos have been widely utilized for the ease of design and synthesis as well as for potential specificity to genes causing diseases. AS-oligos with short length(13 ~ 30 nucleotides) have been designed to bind a complementary sequences by forming Watson-Crick base paring, providing specificity and affinity. Inhibition of gene expression is believed to be achived through either RNaseH activity following formation to DNA-mRNA duplex or sterical hindrance of binding of ribosomal

complex(Dolnick, B. J., Cancer Inv., 9, 185-194, 1991). There also have been some effort to inhibit gene expression by employing trifle helix formation or duplex oligo-decoy mainly aming at or competing with the promoter region of genomic DNA(Young, S. L. et al., Proc. Natl. Acad. Sci. USA, 88, 10023-10026, 1991).

Efficacy of AS-oligos has been validated in some animal models as well as in some of recent clinical studies for human diseases. Intravenous injection of phosphorothioate(hereinafter, referred to as 'PS') AS-oligos for 10 days has eliminated virus DNA of hepatitis B from the duck liver(Offenserger, W. B. et al., EMBO J., 12, 1257-1262, 1993). AS-oligos to angiotensiongen has been found effective to lower blood pressure when injected in spontaneously hypertensive inbred rats(Tomita, N. et al., Hypertension, 26, 131-136, 1995). A subcutaneous application of phosphorothioate AS-oligo against RI, subunit of protein kinase A in nude mice has stopped tumor growth(Nesterova, M. et al., Nat. Med., 1, 528-533, 1995). Several clinical trials using AS-oligos to different genes causing various diseases are also in progress with some results in ovarian cancer and Crohn's disease(Roush, W., Science, 276, 1192-1193, 1997).

However, high expectation for an AS-oligo taking advantages of its sequence specificity for a gene and thus potentialy for a disease have frequently met with disappointments as results from many researchers have not always unambiguous and they were at times contradicting. Salient problems for an AS-oligo were instability to nucleases and inefficient cellular uptake.

Stability of an AS-oligo have been improved to a certain extent by either using modified oligos such as PS- and methylphosphonate(hereinafter, referred to as 'MP')-oligos that are utilized to augment stability against nucleases. However, each of the modified nucleotides exposed problems of its own, those are lack of sequence specificity and insensitivity to RNaseH. Further, there is lingering apprehension for introduction of unwanted mutations upon recycling of the hydrolyzed nucleotides.

AS-oligos bind to complementary target sequences to be effective. All sequences in mRNA have not been found to be equally accessible to AS-oligos. Unequal binding of an AS-oligo could be explained, at least in part, by secondary and/or tertiary structures of target mRNA(Gryaznov, S. et al., Necleic Acids Res., 24, 1508-1514, 1996). Thus, it is conceivables that a region with a less secondary structure could be

targeted readily for an AS-oligo.

In an effort to enhance stability of AS-oligos, the present inventors have devised a rational way of searching better target sites using computer simulation by which secondary structures of mRNA are predicted, so they construct AS-oligos with a stem-loop structure or covalently-closed multiple antisense sequences.

The AS-oligos to c-myb gene could be used for inhibition of tumor cell growth.

The Myb protein, encoded by the c-myb protooncogene, is located mainly inside the nucleus and functions as a transcriptional regulator for G1/S phase transition during the cell cycle. Protooncogene c-myb plays an important role in proliferation and differentiation of hematopoietic cells. Hematopoietic cells exhibit differential expression of c-myb and show little expression of the gene when differentiated to term(Melani, C. et al., Cancer Res., 51, 2897-2901, 1991). C-myb has often been found to be overexpressed in leukemic cells.

It is reported that blockage of c-myb expression by AS-oligos inhibits growth of a promyelocytic cancer cell line HL-60 and a chronic myelogenous leukemia cell line K562(Kimura, S. et al., Cancer Res., 55,

1379-1384, 1995). However, the c-myb AS-oligo used in the above experiments is demonstrated to be partially effective. The c-myb AS-oligo employed for the above experiments is either a phosphodiester(hereinafter, referred to as 'PO')-oligo or a PS capped-oligo(Anfossi, G. et al., Proc. Natl. Acad. Sci. USA, 86, 3379-3383, 1989). These oligo molecules are not stable, especially for the PO-oligo, possibily explaining the partial antisense effect.

AS-oligos selected by rational target site search combined with improved stability would be employed for complete ablation of c-myb mRNA, leading to better inhibition of leukemic cell growth. Recently, a great deal of interest has been focused on developing molecular therapeutics based on AS-oligo strategies against human malignancies. Thus, it is desired to find an improved c-myb antisense molecule which could block lekemic cell growth to completion.

Therefore, to develop AS-oligo of a novel structure with better stability and antisense effect, the present inventors selected 8 sites along c-myb mRNA from secondary structure analysis in the preferred embodiment and combined antisense sequences of the selected c-myb to construct novel large antisense molecules, a covalently-closed multiple

antisense(hereinafter, referred to as 'CMAS')-oligo and a ribbon type antisense (hereinafter, referred to as 'RiAS')-oligo, with loops and a stem structure. Thus, the present inventors have demonstrated that the novel AS-oligos are stable to nuclease activities and show a significant specificity to repress gene expression.

SUMMARY OF THE INVENTION

It is an object of this invention to provide novel AS-oligos containing one or more antisense sequences to mRNA regions with a less secondary structure to improve its target sequence specificity and stability to nuclease activities.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

In such aspects of this invention, the present invention provides antisense sequences selected from mRNA region of c-myb, c-myc, or k-ras with a less secondary structure.

The present invention provides a covalently-closed multiple antisense(CMAS)-oligo containing multiple antisense sequences to c-myb

mRNA. The CMAS-oligo is constructed to form a closed type by ligation using complementary primer.

The present invention also provides a ribbon-type antisense(RiAS)-oligo containing multiple antisense sequences to c-myb mRNA. The RiAS-oligo is composed of two loops containing multiple antisense sequences and a stem connecting the two loops that is constructed by ligation using complementary sequences at both 5 prime ends. In addition, the present invention provides the RiAS-oligos containing multiple antisense sequences to c-myc mRNA or k-ras mRNA.

The present invention further provides pharmaceutical composition containing the novel types of AS-oligos for treatment cancer, immune diseases, infectious diseases, and other human diseases caused by aberrant gene expression..

Further objects and advantages of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a scheme for the construction of a c-myb CMAS-oligo.

FIG. 2 shows electrophoretic mobility patterns of a CMAS-oligo.

A is oligos analyzed by 5% Metaphor agarose gel, where

lane 1; size marker, lane 2; 14 mer ligation primer, lane 3; liner 60 mer oligo, and lane 4; CMAS-oligo.

B shows stability of linear and covalently closed oligos on denaturing polyacrylamide gel, where

lane 1 and 3; no treatment with exonuclease III, and lane 2 and 4; treatment with exonuclease III.

FIG. 3 shows a scheme for the construction of a c-myb RiAS-oligo.

FIG. 4 shows elextrophoretic mobility patterns of a RiAS-oligo.

A is oligos analyzed by a 15% denaturing poilyacrylamide gel, where

lane 1; 58 mer MIJ-78 molecule, and lane 2; 116 mer RiAS-oligo.

B shows stability test of MIJ-78 and a RiAS-oligo upon treatment with exonuclease III, where

lane 1 and 3; no treatment with exonuclease III, and lane 2 and 4; treatment with exonuclease III.

FIG. 5 shows degradation patterens of linear and CMAS-oligos in the presence of serum.

A shows stability test of linear AS-oligo, where

lane 1; no treatment with serum(negative control), lane 2 ; treatment with 50% raw serum, lane

3 ; FBS, and lane 4 ; CS for 24hr respectively.

B shows stability test of CMAS-oligos, where lane 1; no treatment with serum(negative control), lane 2 ; treatment with 50% raw serum, lane 3 ; FBS, and lane 4 ; CS for 24hr respectively.

FIG. 6 shows degradation patterens of linear and RiAS-oligos in the presence of serum.

A shows stability test of MIJ-78 molecules, where lane 1; no treatment with serum(negative control), lane 2 ; treatment with 50% raw serum, lane 3 ; FBS, and lane 4 ; CS for 24hr respectively.

B shows stability test of RiAS-oligos, where lane 1; no treatment with serum(negative control), lane 2 ; treatment with 50% raw serum, lane 3 ; FBS, and lane 4 ; CS for 24hr respectively.

FIG. 7 shows an effect of c-myb CMAS-oligo on c-myb expression in HL-60 cells.

A shows RT-PCR which is performed with total RNA and two c-myb primers, where lane 1; 60 mer CMAS-oligo 0.3 ug + Lipofectin 1 ug, lane 2; 60 mer CMAS-oligo 1 ug + Lipofectin 1 ug, and lane 3; scrambled AS-oligo 1 ug + Lipofectin 1 ug.

B shows RT-PCR which is performed with total RNA and two c-myb primers, where upper panel; the hybridized RT-PCR bands of c-myb mRNA, and lower panel; the hybridized RT-PCR bands of

β-actin mRNA.

FIG. 8 shows an effect of c-myb RiAS-oligo on the c-myb mRNA expression in HL-60 cells.

A shows RT-PCR which is performed with total RNA using two c-myb primers, where

lane 1; RiAS-oligo 0.1 ug + Lipofectin 0.8 ug, lane 2; RiAS-oligo 0.2 ug + Lipofectin 0.8 ug, and lane 3; SC-oligo 0.2 ug + Lipofectin 0.8 ug.

B shows RT-PCR which is performed with total RNA and two c-myb primers, where

upper panel; the hybridized RT-PCR bands of c-myb mRNA, and lower panel; the hybridized RT-PCR bands of β-actin mRNA.

FIG. 9 shows an effect of 60 mer CMAS or linear AS-oligo on proliferation of HL-60 cells, where

-◆ -; CMAS-oligos(1), -■ -; CMAS-oligos(2), -● -; AS-oligos, -▲ -; S-MIJ-7, ● ; Lipofectin alone, ○ ; untreated control, and (1) or (2); times of treatement with AS-oligos.

FIG. 10 shows an effect of c-myb RiAS-oligo on proliperation of HL-60 cells.

A shows MTT assay of a c-myb RiAS-oligo.

B shows [^{3}H] thymidine incorporation of a c-myb RiAS-oligo.

FIG. 11 shows a photomicrograph for inhibition of HL-60 cells with c-myb RiAS-oligo.

A is c-myb RiAS-oligo, B is SC-oligo, and C is Lipofectin alone.

FIG. 12 shows a photomicrograph for inhibition of HT-29 cells with c-myb RiAS-oligo.

A is c-myb RiAS-oligo, B is SC-oligo, and C is Lipofectin alone.

FIG. 13 shows a photomicrograph for inhibition of HT-29 cells with c-myc RiAS-oligo.

A is c-myc RiAS-oligo, B is SC-oligo, and C is Lipofectin alone.

FIG. 14 shows a photomicrograph for inhibition of HT-29 cells with k-ras RiAS-oligo.

A is k-ras RiAS-oligo, B is SC-oligo, and C is Lipofectin alone.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides novel AS-oligos containing one or more antisense sequence to regions with a less secondary structure.

Particulary, in the preferred embodiment, 8 different regions of c-myb mRNA, one of protooncogenes, for target sites of antisense oligos

were selected. The rational taregt site search for an AS-oligo is employed to improve the chance to predict a natural secondary structure. The above 8 antisense sequences are complementary to the selected target sites. Among the 8 selected target sites for AS-oligos, 4 sites(MIJ-1, MIJ-2, MIJ-3, and MIJ-4) are finally chosen in a combination upon forming a CMAS molecule and a 3 sites(MIJ-3, MIJ-4, and MIJ-17) in a combination forming a RiAS molecule as they form minimal intramolecular secondary structure(see Table 1).

AS-oligos having phosphodiester backbone lacked stability which was essential for successful antisense application. Modified oligos, such as PS-oligo or MP-oligo, exhibited improved stability, but the gain in stability was only partial and beared potential harzard misincorporation of the hydrolyzed modified-nucleotides during DNA replication or repair. It was previously reported that stem-loop oligos complexed with cationic liposomes also showed partial improvement of stability. However, stability still remains a major concerns for AS-oligos. So, these inventors tried to develop an improved AS-oligo containing better stability.

Therefore, the present invention provides a

covalently-closed multiple antisense(CMAS)-oligo.

Particulary, intracellular secondary structure of AS-oligos was constructed without duplex formation between an AS-oligo and target mRNA. In the preferred embodiment, these AS-oligos are designated a form of a CMAS(covalently-closed multiple antisense)-oligo containing four antisense sequences(MIJ-1, MIJ-2, MIJ-3, and MIJ-4) which is described by SEQ ID NO : 1, NO : 2, NO : 3, and NO : 4 in a loop are placed in tandem to increase the length of CMAS-oligos(see FIG.1). The CMAS-oligos show electrophoretic mobility patterns that it is slowed by about 10% than its linear presusor on a 15% denaturing PAGE gel(see A of FIG. 2). The CMAS-oligos are, as expected, resistant to exonuclease III and are shown in multiple bands on a denaturing PAGE gel, with monomer(60 mer) being the most abunbant, dimers(120 mer) and trimers(180 mer). In contrast to a CMAS-oligo, linear oligos are completely degraded after 2 hr incubation with exonuclease III(see B of FIG. 2).

The present invention also provides a ribbon-type antisense(RiAS)-oligo.

The CMAS-oligo, although very stable, needs a primer for intramolecular ligation that must be eliminated afterward. So, to avoid using a ligation

primer and obtain a homologus population of AS-oligo, these inventors makes two AS-oligos enzymatically ligated to form a ribbon-type closed molecule termed a RiAS-oligo.

The RiAS-oligo(116 mer) consists of two loops and one stem connecting two loops(see FIG. 3). In the preferred embodiment, three antisense sequences(MIJ-3, MIJ-4, and MIJ-17) which is described by SEQ IN NO : 3, NO : 4, and NO : 5 in a loop are placed in tandem to increase the length of RiAS-oligo. Consequently, two copies of three different antisense sequences(total 6 antisense sequences) are in the RiAS-oligo. This enlarged length of the loop in RiAS-oligo is to accommodate torsional stress caused by forming a duplex with the target mRNA sequences. The RiAS-oligo is found to be slowed markedly than its linear precusor(MIJ-78) on a denaturing PAGE gel(see A of FIG. 4). The RiAS-oligo is, as expected, resistant to exonuclaese III and is shown in a major band(116 mer) on a PAGE gel. In contrast to the RiAS-oligo, MIJ-78 is completely degraded after 2 hr incubation with exonuclease III(see B of FIG. 4).

To demonstrate the enhanced stability of the CMAS-oligo and the RiAS-oligo of this invention against nuclease avtivities, the CMAS-oligo and the

RiAS-oligo are incubated with serums that are not heat inactivated to maintain nuclease activities.

As a result, linear 60 mer oligo(precusor of the CMAS-oligo, see A of FIG. 5) and linear 59 mer oligo(precusor of the RiAS-oligo, see A of FIG. 6) are completely digested after 24 hr incubation in the presence of serum. The CMAS-oligo and the RiAS-oligo, however, are remained mostly intact after 24 hr incubation with raw human serum, FBS, and calf serum, exhibiting significantly improved stability that does the linear one against nucleases activities(see B of FIG. 5 and B of FIG. 6).

In addition, it is demonstrated that the CMAS-oligo functions well eliminating target mRNA in a sequence specific manner.

Particulary, the CMAS-oligo was combined with Lipofectin to deliver into cells. Lipofectin is employed as it is found to be less toxic to cells and yield consistent results. MIJ-5, the CMAS-oligo to human c-myb, is able to reduce more than 95% of c-myb mRNA when compared to a control SC-oligo. Meanwhile, the linear counterpart of MIJ-5, MIJ-5A, decreases some 37% of c-myb mRNA(see A of FIG. 7). These results indicate that the CMAS-oligo of this invention is superior to linear one in ablating target mRNA even

when used in a smaller amount.

The RiAS-oligo functions of eliminating target mRNA was demonstrated by the same method in the CMAS-oligo.

HL-60 cells were tested with RiAS-oligos, scrambled(SC)-oligos as well as Lipofectin alone. The RiAS-oligo is delivered into cells after forming a complex with Lipofectin. Consequently, the RiAS-oligo is able to ablate c-myb mRNA to completion. In contrast, SC-oligo exhibits a mild reduction of c-myb mRNA by about 30% when compared to Lipofectin treatment alone(see A of FIG. 8). These results indicate that the RiAS-oligo of this invention is excellent in ablating target mRNA even when used in a small amount.

The present inventors also examines antisense effect of the CMAS-oilgo and the RiAS-oligo by Southern blotting of the PCR product.

In case of the CMAS-oligo, when c-myb meassage is amplified with RT-PCR, more than 90% of the meassage is found to be reduced with treatment of MIJ-5(see B of FIG. 7).

In RiAS-oligo, c-myb meassage amplified by RT-PCR is detected with a labeled internal hybridization oligo(30 mer)(B of FIG. 8). The result confirms that the amplified meassage is indeed c-myb derived.

The present invention also provides pharmaceutical composition containing the novel types of AS-oligos for treatment cancer, immune diseases, infectious diseases, and other human diseases caused by aberrant gene expression.

It is demonstrated that the c-myb CMAS-oligo or the c-myb RiAS-oligo inhibits leukemic cell growth. Particulary, growth inhibition of the c-myb CMAS-oligo and the c-myb RiAS-oligo to leukemic cells was measured by three methods, MTT assay, [^{3}H] thymidine incorporation or colony formation on soft agarose.

As a result of MTT assay, cell number is reduced progressively when treated with increasing amounts of MIJ-5, the CMAS-oligo to human c-myb. Inhibition of cell growth is more pronounced when cells are treated twice with MIJ-5. More than 80% of growth inhibition of HL-60 cells is observed even at a low concentration(see FIG. 9). Meanwhile, the linear 60 mer AS-oligo, MIJ-5A, and linear sense oligo does not bring about any significant inhibition of cell growth when compared with a sham control. These results indicate that the c-myb CMAS-oligo of this invention is an effective antisense agent and is efficacious against tumor growth in a concentration dependent manner.

In addition, cell growth is observed to be

inhibited by 91% with the RiAS-oligo(see A of FIG. 9). Meanwhile, the SC-oligo and Lipofectin alone does not significantly inhibit cell growth when compared to that of the untreated control. These results indicate that the c-myb RiAS-oligo of this invention is also an effective antisense agent for inhibition of leukemic cell growth.

In colony formation on soft agarose, MIJ-5 reduces the number of colonies formed by more than 90%(see Table 2). MIJ-5A also reduces colonies formed but less effective for growth inhibition, about 70% reduction of colonies. On the other hand, a sense oligo and a SC-oligo exhibits marginal reduction of colonies, by about 11% and 32% respectively.

Also, the c-myb RiAS-oligo transfected into cells is able to reduce the number of colonies formed by about 92%(see Table 3) when compared to an untreated control. Meanwhile, a SC-oligo and Lipofectin alone exhibits marginal reduction of colonies, by about 7.9% and 7.1% respectively.

In addition, it is observed growth inhibition of the c-myb RiAS-oligo to leukemic cells by [^{3}H] thymidine incorporation. Particulary, the RiAS-oligo inhibits growth of HL-60 cells by 93%. Meanwhile, the

SC-oligo and Lipofectin alone exhibits mild inhibition of cell growth, by about 16.8% and 15.4% respectively (see B of FIG. 10). On a microscopic observation, after treated with the c-myb RiAS-oligo, growth of HL-60 cells are markedly inhibited when compared with cells treated with scrambled oligo and Lipofectin alone(see FIG. 11).

Encouraged by the remarkable inhibition activity of c-myb RiAS-ologo in this invention, these inventors construct other RiAS-oligos against two different protooncogenes, c-myc and k-ras, and examine if the c-myc RiAS-oligo and the k-ras RiAS-oligo functiones well in inhibiting cell growth.

As a result of microscopic observations, growth of HT-29 cells is markedly inhibited by all RiAS-oligos, c-myb RiAS-oligo, c-myc RiAS-oligo, and k-ras RiAS-oligo, when compared with cells treated with scrambled oligos and Lipofectin alone(see FIG. 12, FIG. 13, and FIG. 14)

Therefore, the novel RiAS-oligos of the present invention show effective growth inhibition of tumor cells to various target sequences as well as enhanced stability to nuclease activity. So, the novel RiAS-oligos of this invention may be effectively employed for developing molecular antisense oligos to treat various human diseases.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1 : Selection of target sites for an AS-oligo

Target site selection of an AS-oligo had been found to be critical to achive antisense effect, reduction or ablation of target mRNA. However, the approach for the target site selection had been rather arbitrary. So, these inventors scanned the entire sequence of human c-myb mRNA for putative secondary structures to search a rational target site in the preferred embodiment.

Particulary, simulation of secondary structures was carried out with the DNAsis program(Hitach Software, Japan). Entire c-myb sequence was scanned sequentially for secondary structure formation in contiguous frames of 100 bases. Then, frames for the simulation of secondary structure were staggered down by 30 bases, resulting in an overlap of 60 bases on

the 5 prime side. This process was repeated again such that any given sequence was scanned for its potential secondary structure in three different frames.

As a result, eight sequences which had minimal secondary structures in three different frames were selected from the c-myb mRNA sequence(Table 1). The rational taregt site search for an AS-oligo was employed to improve the chance to predict a natural secondary structure.

TABLE 1. Eight target sequences for antisense oligos selected from the c-myb mRMA sequences

Name	Complementary site	Type	Size (mer)	Sequence
MIJ-1	253-267	Antisense	15	SEQ ID NO:1
MIJ-2	401-415	Antisense	15	SEQ ID NO:2
MIJ-3	613-627	Antisense	15	SEQ ID NO:3
MIJ-4	1545-1559	Antisense	15	SEQ ID NO:4
MIJ-6	253-267	Antisense	15	SEQ ID NO:9
MIJ-16	585-602	Antisense	18	SEQ ID NO:10
MIJ-17	961-978	Antisense	18	SEQ ID NO:5
MIJ-19	97-114	Sense	8	SEQ ID NO:11

As illustrated in the table 1, the above 8

antisense sequences were complementary to the selected target sites. Among the 8 selected target sites for AS-oligos, 4 sites(MIJ-1, MIJ-2, MIJ-3, and MIJ-4) were finally chosen in a combination upon forming a CMAS molecule and a 3 sites (MIJ-3, MIJ-4, and MIJ-17) in a combination forming a RiAS-oligo as they form minimal intramolecular secondary structure.

Example 2 : Construction of a covalently-closed multiple antisense(CMAS)-oligo

These inventors tried to develop an improved AS-oligo containing better stability.

4 different AS-oligos(MIJ-1, MIJ-2, MIJ-3, and MIJ-4) obatined by Example 1 was used to construct a CMAS-oligo. To bind to target sites more readily, one CMAS-oligo was constructed to harbor 4 different antisense sequences in a combination with the least secondary structure. AS-oligos were phosphorylated during synthesis at the 5 prime end to follow intra- or intermolecular covalent ligations(FIG. 1). The sequence of the 60 mer AS-oligo containing 4 different antisense sequences was described by SEQ. ID NO : 7. Both ends of the AS-oligo was joined with a ligation primer which has complementary sequences in its both halves to the both extreame end sequences(7 bases on each side) of the 60 mer AS-oligo. The sequence of

the 14 mer ligation primer was described by SEQ ID NO : 6. Ligation primer was mixed with AS-oligo and was heated at 85°C for 2 min followed by gradual cooling to ambient temperature. One unit of T4 ligase was added and incubated at 16°C for 16 hr to generate a covalently-closed molecule. The CMAS-oligo was electropheoresed on a 5% Methphor™ agarose gel(FMC, USA) or on 12% denaturing PAGE and identified for its resistance to exonuclease III as well as for slight gel retardation compared with the liner 60 mer oligos. Ligation primer was degraded with exonuclease III or detached from the CMAS-oligo by running on a denaturing gel after heating the oligos at 90°C.

Consequently, intracellular secondary structure of an AS-oligo was constructed without duplex formation between an AS-oligo and target mRNA. This AS-oligo was designated a CMAS(covalently-closed multiple antisense)-oligo. The CMAS-oligo showed electrophoretic mobility patterns that it was slowed by about 10% than its linear presusor on a 15% denaturing PAGE gel(A of FIG. 2). The CMAS-oligo was, as expected, resistant to exonuclease III and was shown in multiple bands on a denaturing PAGE gel, with monomer(60 mer) being the most abunbant, then dimers(120 mer) and trimers(180 mer)(B of FIG. 2). In contrast to the CMAS-oligo, a linear oligo was

completely degraded after 2 hr incubation with exonuclease III.

Example 3 : Construction of a ribbon-type antisense(RiAS)-oligo

These inventors made two AS-oligos enzymatically ligated to form a ribbon-type closed molecule termed a RiAS-oligo.

Particulary, the RiAS-oligo consisted of two loops and one stem connecting the two loops. Each loop contained three different antisense(MIJ-3, MIJ-4, and MIJ-17) sequences that were described by SEQ ID NO : 3, NO : 4, and NO : 5. To bind to target sites more readily, a combination of three antisense sequences with a least possible secondary structure was chosen for the AS-oligo. C-myb AS-oligo(MIJ-78) was phosphorylated at the 5 prime end. Sequences of the 58 mer MIJ-78 was described by SEQ ID NO : 8. MIJ-78 was to form a stem-loop structure. The stem was formed by complementary sequences at both ends of each oligo. The 5 prime terminus of the stem had 4 bases of a single stranded sequense of 5'-(p)GATC-3'. Two MIJ-78 molecules were joined by the complementary 4 base sequences at both 5 prime ends. MIJ-78 molecules were mixed and heated to 85°C for 2 min followed by gradual cooling to ambient temperature. One unit of

T4 DNA ligase was added and incubated at 16oC for 24 hr to generate a covalently ligated molecule with diad-symmetry(FIG. 3). The RiAS-oligo was electrophoresed on 15% denaturing polyacrylamide gel and examined for its resistance to exonuclease III as well as for gel retardation.

As a result, the RiAS-oligo(116 mer) consisting of two loops and one stem connecting two loops was constructed. Three antisense sequences in a loop were placed in tandem to increase the length of the RiAS-oligo. Consequently, two copies of three different antisense sequences(total 6 antisense sequences) were in the RiAS-oligo. This enlarged length of the loop in the RiAS-oligo was to accommodate torsional stress caused by forming a duplex with the target mRNA sequences. The RiAS-oligo was found to be slowed markedly than its linear precusor(MIJ-78) on a denaturing PAGE gel(A of FIG. 4). The RiAS-oligo was, as expected, resistant to exonuclaese III and was shown in a major band(116 mer) on a PAGE gel(B of FIG. 4). In contrast to RiAS-oligo, MIJ-78 was completely degraded after 2 hr incubation with exonuclease III.

Example 4 : Enhanced stability of the CMAS-oligo and the RiAS-oligo to nuclease activities

In order to test stability of the CMAS-oligo and the RiAS-oligo of this invention against nuclease activities, the CMAS-oligo and the RiAS-oligo was incubated with serums that were not heat inactivated to maintain nuclease activities.

Particulary, one ug each of the nonspecific control-phosphodiester oligo(liner 60 mer) and the CMAS-oligo were incubated with either raw human serum, FBS and calf serum(non-heat inactivated; HyClone, Logan, Utah, USA) or exonuclease III. 15% of each serum was added to AS-oligos in an 100 ul reaction volume and incubated at 37°C for 24 hr. AS-oligos were then extrected with phenol and chloroform, and were examined on 15% denaturing PAGE gel. Exonuclease III(Takara, Japan) at 160 U/ug oligo was added to linear and CMAS-oligos and incubated at 37°C for 2 hr. AS-oligos treated with exonuclease III were also extracted and electrophoresed in the same manner.

As a result of CMAS-oligo, liner 60 mer oligo was completely digested after 24 hr incubation in the presence of serum(A of FIG. 5). The CMAS-oligo of this invention, however, was remained mostly intact after 24 hr incubation with raw human serum, FBS, and calf serum, exhibiting significantly improved stability than the linear one against nucleases(B of FIG. 5).

In the case of the RiAS-oligo, linear 58 mer was completely hydrolyzed after 24 hr incubation in the presence of each different serum(A of FIG. 6). The RiAS-oligo of this invention, however, remained mostly intact after 24 hr incubation with the raw serums, exhibiting significantly improved stability than the linear one against nucleases(B of FIG. 5).

Example 5 : Specific reduction of c-myb mRNA by the CMAS-oligo and the RiAS-oligo

Encouraged by the remarkable stability of the CMAS-oligo and the RiAS-oligo in this invention, these inventors examined if the AS-oligo functioned well in eliminating target mRNA in a sequence specific manner.

<5-1> Cell lines and tissue culture

Leukemic cell lines, HL-60(promyelocyte leukemic cell line) and K562(chronic myelogenous leukemic cell line), were obtained from ATCC(American Type Culture Collection, USA) and cultured in RPMI 1640(Gibco BRL, USA) supplemented with 10% heat-inactivated FBS(HyClone, USA) and 1% penicillin/streptomycine. Cells were maintained in a CO_2 incubator at 37°C. Routine cell culture practices were strictly adhered

to keep proper cell density and to avoid cells cultured more than 5 generations after thawing stock vials. Culture media were exchanged a day before treating with AS-oligos.

<5-2> Transfection of the CMAS-oligo and the RiAS-oligo complexed with cationic liposomes

0.3 ug CMAS-oligo plus 0.8 ug LipofectinTM(Gibco BRL, USA) or 0.2 ug RiAS-oligo plus 0.8 ug Lipofectin™ were dilluted in 20 ul OPTI-MEM™ seperately and incubated at ambient temperature for 40 min. Each component was then mixed to form a complex at ambient temperature for 15 min. Cells were added with fresh culture media without antibiltics(RPMI 1640 + 10% FBS) 1 day prior to adding oligos and washed twice with OPTI-MEM before an experiment. Cell density was adjusted to 5 X 10^5 cells/ml and aliquoted in 100 ul each in a 48-well plate(Falcon, USA). 40 ul of liposome-oligo complex was added to cells twice, once on day 0 and once on day 1. Cells treated with oligos were incubated at 37°C and 5% CO_2 for 4 hr and then added 100 ul of OPTI-MEM with 10% FBS. The next day, 100 ul of supernatant was carefully removed and replaced with 20 ul of fresh OPTI-MEM containing oligo-liposome complex. Four hours later, cells were added with additional 100 ul of complete media with

antibiotics and incubated at 37°C 1 more day before assay.

<5-3> Isolation of total RNA and RT-PCR

Total RNA was isolated with Tripure™ Isolation Reagent(Boehringer Manhein, Germany) according to the procedure recommended by the menufacturer. Briefly, cells harvested were added with 0.4 ml Tripure reagent, 10 ug glycogen and 80 ul chloroform to obtain total RNA. RT-PCR was performed in a single reaction tube with Access™ RT-PCR kit(Promrga, USA). In a PCR tube were added RNA, PCR primers, AMV reverse transcriptase(5 U/ul), Tfl DNA polymerase(5 U/ul), dNTP(10 mM, 1 ul) and $MgSO_4$(25 mM, 2.5 ul). Synthesis of the first strand cDNA was done at 48 °C for 45 min in a DNA termal cycler(Hybaid, USA). 25 cycles of PCR amplification were subsequently carried out with the recommended condition by the manufacturer. Amplified PCR product was confirmed in an 1% agarose gel and quantitation was done with a gel documentation program(Bio-Rad, USA).

<5-4> Sourthern hybridization of RT-PCR fragments

RT-PCR products were electrophoresed on an 1% agarose gel. DNA was transferred onto a nylon membrane(New England Biolab, USA) for 4 hr in 0.4 M

NaOH. The membrane was hybridized with 30 mer internal primer labeled with ECL 3 prime end oligo-labeling and detection system(Amersham Life Science, England). The sequence of 30 mer internal primer was described by SEQ ID NO : 9. Hybridization was carried out at 62 °C for 60 min in 6 ml buffer containing 5 X SSC, 0.02% SDS. The membrane was washed twice in 5 X SSC containing 0.1% SDS and washed twice with 1 X SSC containing 0.1% SDS at for 15 min. The membrane was blocked with a blocking solution and then treated with HRP(horse radish peroxidase) anti-fluorescein conjugated antibody for 30 min before autoradiography.

It was demonstrated that the CMAS-oligo of this invention functioned well eliminating target mRNA in a sequence specific manner.

Particulary, the CMAS-oligo was complexed with Lipofectin to deliver into cells. Lipofectin was employed as it was found to be less toxic to cells and yield consistent results. As a result, 0.3 ug MIJ-5, a CMAS-oligo to human c-myb, was complexed with 1 ug Lipofectin for transfection into HL-60 cells. MJ-5 was able to reduce more than 95% of c-myb mRNA when compared to a control SC-oligo. Meanwhile, the linear counterpart of MIJ-5, MIJ-5A, decreased some 37% of

c-myb mRNA(A of FIG. 7). These results indicated that the CMAS-oligo of this invention is superior to linear one in ablating target mRNA even when used in a smaller amount.

It was also demonstrated that the RiAS-oligo of this invention functioned well eliminating target mRNA in a sequence specific manner, either.

HL-60 cells were transfected with the RiAS-oligos, SC-oligos as well as Lipofectin alone. The RiAS-oligo was delivered into cells after forming a complex with Lipofectin. The RiAS-oligo(0.1 ug or 0.2 ug) to human c-myb was complexed with 0.8 ug Lipofectin for transfection into HL-60 cells. Consequently, 0.2 ug of the RiAS-oligo(40 nM) was able to ablate c-myb mRNA to completion. Meanwhile, 0.1 ug of the RiAS-oilgo decreased about 70% of c-myb mRNA(A of FIG. 8). In contrast, SC-oligo exhibited a mild reduction of c-myb mRNA by about 30% when compared to Lipofectin treatment alone. However, β-actin expression shown in the bottom panel was not affected by the treatment of the RiAS-oligo as well as other treatment conditions. These results indicated that the RiAS-oligo was excellent in ablating target mRNA even when used in a small amount.

The present inventors also examined antisense

effect of the CMAS-oilgo and the RiAS-oligo by Sourthern blotting with PCR products. HL-60 cells were transfected with oligos including MIJ-5 and control oligos, and the cells were used to isolate total DNA.

In case of the CMAS-oligo, when c-myb meassage was amplified with RT-PCR, more than 90% of the meassage was found to be reduced with treatment of MIJ-5(B of FIG. 7). However, β-actin expression shown in the bottom panel was not affected by the treatment of MIJ-5.

In RiAS-oligo, C-myb meassage amplified by RT-PCR was detected with a labeled internal hybridization oligo(30 mer)(B of FIG. 8). The results confirmed that the amplified meassage was indeed c-myb derived, with total elimination of the meassage by treatment with 0.2 ug of the c-myb RiAS-oligo.

Example 6 : Effective growth inhibition of leukemic cells by the c-myb CMAS-oligo and the c-myb RiAS-oligo

It was reported that c-myb played an important role in proliferation of leukocytes. AS-oligos to c-myb were also reported to block leukemic cell growth preferentially. So, these inventors tested the c-myb CMAS-oligo and the c-myb RiAS-oligo of this invention for inhibiting leukemic cell growth.

Particulary, growth inhibition of the c-myb CMAS-oligo and the c-myb RiAS-oligo to leukemic cells was measured by three methods, MTT assay, [^{3}H] thymidine incorporation or colony formation on soft agarose.

<6-1> MTT assay

For MTT(3,-[4,5-Dimethythiazol-2-yi]2,5-diphenyl-tetrazolium bromide, hereinafter, referred to as 'MTT') assay, HL-60 cells were washed twice with OPTI-MEM and aliquoted in a 96-well plate(5 X 10^3 cells/well) in a 50 ul volume. Cells were treated with performed complex between oligos in different amount(0.01 - 1 ug/15 ul in CMAS-oligo or 0.2 ug/15 ul in RiAS-oligo) and Lipofectin(0.2 ug/15 ul) for 5 hr and cultured for 5 days. Cells were then harvested in an 100 ul volume and added with 20 ul(100 ug) of an MTT reagent(5 mg/ml in PBS; Sigma, USA), followed by 4 hr incubation at 37°C. An 100 ul of isopropanol(containing 0.1 N HCl) was added to the cells and incubated for one more hour at the ambient temperature. Absorbance was measured at 570 nm with an ELISA reader to score the amount of cells survived.

In CMAS-oligo, cell number was reduced progressively when treated with increasing amounts of

MIJ-5. Inhibition of cell growth was more pronounced when cells were treated twice with MIJ-5. More than 80% of growth inhibition of HL-60 cells was observed even at a low concentration, 0.13 ug(total 0.24 ug) of the CMAS-oligo(FIG. 9). Meanwhile, the linear 60 mer AS-oligo, MIJ-5A, and linear sense oligo did not bring about any significant inhibition of cell growth when compared with a sham control. These results indicated that the c-myb CMAS-oligo was an effective antisense agent and was efficacious agent against tumor growth in a concentration dependent manner.

In RiAS-oligo, cell growth was also observed to be inhibited by 91% with the RiAS-oligo(A of FIG. 10). Meanwhile, the SC-oligo and Lipofectin alone did not significantly inhibited cell growth when compared to that of the untreated control. These results indicated that the c-myb RiAS-oligo was an effective antisense agent for inhibition of leukemic cell growth.

<6-2> Colony formation on soft agarose

Growth inhibition of the c-myb CMAS-oligo and the c-myb RiAS-oligo to leukemic cells was also measured by colony formation on soft agarose as another way.

Particulary, K562 cells were transfected as described above in Example 6 and cultured at 37°C and

5% CO_2 for 24 hr. An equal volume mixture of 0.8% low melting agarose and 2 (RPMI 1640 contaning 20% FBS plus antibiotics were added to cells and seeded in a 6 well-plate to solidify. The 6-well plate was cooled to 4°C for 5 min and incubated for 15 days. Colonies containing more than 20 cells were scored as positive.

As a result, CMAS-oligo MIJ-5 reduced the number of colonies formed by more than 90%(Table 2). MIJ-5A also reduced colonies formed but less effective for growth inhibition, about 70% reduction of colonies. On the other hand, a sense oligo and a SC-oligo exhibited marginal reduction of colonies, by about 11% and 32% respectiveiy.

TABLE 2. Effects of c-myb oligos on colony formation of K562 cells

Oligos			Number of colony	Colonies formed %
Structure	Size(mer)	Type		
Linear	15	AS-MIJ-1	55	44.4
Linear	15	S-MIJ-3	110	88.7
Linear	15	SC-MIJ-1	84	67.7
Linear	60	AS-MIJ-5A	29	23.4
CMAS	60	AS-MIJ-5	9	7.2
Lipofectin alone			109	88.0

Untreated control			124	100.0

On the other hand, cells transfected with the c-myb RiAS-oligo was able to reduce the number of colonies formed by about 92%(Table 3) when compared to an untreated control. Meanwhile, a SC-oligo and Lipofectin alone exhibited marginal reduction of colonies, by about 7.9% and 7.1% respectively.

TABLE 3. Effects of c-myb oligos on colony formation of K562 cells

Oligos	Size(mer)	Number of colony	Colonies formed %
RiAS-oligo	116	7.6 ∓ 1.53	7.8
Scrambled oligo	116	0.5 ∓ 2.12	92.1
Lipofectin alone		91.3 ∓ 4.16	92.9
Untreated control		98.3 ∓ 4.04	100.0

<6-3> [^{3}H] thymidine incorporation

Growth inhibition of leukemic cells by the c-myb RiAS-oligo was also measured by [^{3}H] thymidine incorporation.

For [^{3}H] thymidine incorporation, HL-60 cells were treated with AS-oligo as described above. Cells

were added with 0.5 uCi of [^{3}H] thymidine(2.0 Ci/mmol; Amersham, England) and incubated for 16 hr in triplicate. Cells were then harvested on a glass microfiber filter(Whatman GF/C, England). The filter was washed with in the order of cold PBS, 5% TCA and absolute ethanol. [^{3}H] thymidine incorporation was measured with the liquid scintillation counter in a cocktail solution containing toluene, Triton X-100, PPO and POPOP.

Consequently, the RiAS-oligo(0.2 ug) inhibited growth of HL-60 cells by 93%(B of FIG. 10). Meanwhile, the SC-oligo and Lipofectin alone exhibited mild inhibition of cell growth, by about 16.8% and 15.4% respectiverly. On a microscopic observation, after treated with the c-myb RiAS-oligo, growth of HL-60 cells was markedly inhibited when compared with cells treated with scrambled oligo and Lipofectin alone(FIG. 11).

Example 8 : Effective growth inhibition of the c-myc RiAS-oligo and the k-ras RiAS-oligo

Encouraged by the remarkable inhibition activity of c-myb RiAS-ologo in this invention, these inventors constructed other RiAS-oligos against two different protooncogenes, c-myc and k-ras, as the same method in Example 3.

And then, they examined if the c-myc RiAS-oligo and the k-ras RiAS-oligo functioned well in inhibiting cell growth.

Particulary, they used different cell line, colorectal adenocarcinoma cell line HT-29. Growth inhibition of the c-myc RiAS-oligo and k-ras RiAS-oligo to tumor cells was measured by [^{3}H] thymidine incorporation as the same method in Example <6-3>. HT-29 cells were treated with cationic liposome complexes of 0.2 ug c-myb RiAS-oligo plus 0.6 ug Lipofectin or 0.5 ug c-myc RiAS-oligo plus 1.5 ug Lipofectin or 0.5 ug k-ras RiAS-oligo plus 1.5 ug Lipofectin, respectively. After treated respective RiAS-oligos for 5 days, growth of HT-29 cells was observed using microscophy. Each photomicrograph exhibited the effect on growth inhibition after treatement with respective RiAS-oligos(A), scrambled oligo(B), and Lipofectin alone(C).

As a result of microscopic observations, growth of HT-29 cells was markedly inhibited by all RiAS-oligos, c-myb RiAS-oligo, c-myc RiAS-oligo, and k-ras RiAS-oligo, when compared with cells treated with scrambled oligos and Lipofectin alone(FIG. 12, FIG. 13, and FIG. 14)

Therefore, the novel RiAS-oligos of the present invention showed effective growth inhibition of tumor

cells to various target sequences as well as enhanced stability to nuclease activity. So, the novel RiAS-oligos of this invention might be effectively employed for developing molecular antisense oligos to treat various human diseases.

INDUSTRIAL APPLICABILITY

The present invention provides novel AS-oligos containing one or more antisense sequence to mRNA region with a less secondary structure and having better target sequence specificity and stability to nuclease activities.

Particulary, the present invention provides a covalently-closed multiple antisense(CMAS)-oligo containing multiple target antisense sequences to c-myb mRNA which is constructed to form a closed type by ligation using complementary primer. In addition, the present invention provides a ribbon-type antisnese(RiAS)-oligo containing multiple target antisense sequences to c-myb mRNA which is constructed to form a stem-loop structure by ligation using complementary sequences at both 5 prime ends.

It is demonstrated that aberrant gene expression is effectively ablated by the novel AS-oligos of this invention when human tumor cells are treated with the c-myb RiAS-oligo and the c-myb CMAS-oligo as well as c-myc RiAS-oligo and k-ras RiAS-oligo. Thus, it suggests that the novel AS-oligos of this invention may be employed for developing molecular antisense drugs to various genes causing diseases as well as for the functional study of a gene. Particulary, the

novel AS-oligos of this invention may be used for developing pharmaceutical composition for treatment cancer, immune diseases, infectious diseases, or other human diseases caused by aberrant gene expression.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is Claimed is

1. A novel antisense oligo which improve its target sequences specificity by containing one or more antisense sequence to mRNA regions with less secondary structures and improve its stability to nuclease activities by constructing closed type.

2. The novel AS-oligo according to claim 1, wherein the mRNA is transcribed from various genes causing human diseases.

3. The novel AS-oligo according to claim 1, which is a covalently-closed multiple antisense(CMAS)-oligo, wherein CMAS-oligo is constructed into a closed type by ligation of the AS-oligo using a ligation primer.

4. The CMAS-oligo according to claim 3, wherein the AS-oligo is 60 mer AS-oligo described by SEQ. ID NO : 7.

5. The CMAS-oligo according to claim 4, wherein the SEQ. ID NO : 7 comprises 4 antisense sequences described by SEQ ID NO : 1, NO : 2, NO : 3, and NO : 4.

6. The CMAS-oligo according to claim 3, wherein the ligation primer comprises 14 mer nucleotides described by SEQ. ID NO : 6.

7. The CMAS-oligo according to claim 3, which is effective for ablating or reducing aberrent gene expression involved in human diseases.

8. The CMAS-oligo according to claim 7, wherein the gene is protooncogene c-myc, c-myb, or k-ras.

9. The CMAS-oligo according to claim 3, which inhibits effectively tumor cell growth.

10. The CMAS-oligo according to claim 9, wherein the tumor cell is promyelotic leukomic cell line HL-60, chronic myelogenous leukemic cell line K562, or colorectal adenocarcinoma cell line HT-29.

11. The novel AS-oligo according to claim 1, which is a ribbon-type antisense(RiAS)-oligo, wherein the RiAS-oligo is constructed into a ribbon type by ligation of two AS-oligos using complementary sequences at each 5 prime ends.

12. The RiAS-oligo according to claim 11, which has a stem-loop structure of two loops and one stem connecting two loops.

13. The RiAS-oligo according to claim 11, wherein the AS-oligo is 58 mer AS-oligo described by SEQ. ID NO : 8.

14. The RiAS-oligo according to claim 13, wherein the SEQ. ID NO : 8 comprises 3 antisense sequences described by SEQ. ID NO : 3, NO : 4, and NO : 5.

15. The RiAS-oligo according to claim 11, wherein the complementary sequences are described in 5'-(p)GATC-3'.

16. The RiAS-oligo according to claim 11, which is effective for ablating or reducing aberrent gene expression involved in human diseases.

17. The RiAS-oligo according to claim 11, wherein the gene is protooncogene c-myc, c-myb, or k-ras.

18. The RiAS-oligo according to claim 11, which inhibits effectively tumor cell growth.

19. The RiAS-oligo according to claim 18, wherein the tumor cell is promyelotic leukomic cell line HL-60, chronic myelogenous leukemic cell line K562, or colorectal adenocarcinoma cell line HT-29.

20. AS-oligo-liposome complex containing the CMAS-oligo of claim 3 or the RiAS-oligo of claim 11.

21. The AS-oligo-liposome complex according to claim 20, wherein the liposome is a cationic liposome.

22. Pharmaceutical composition containing the CMAS-oligos of claim 3 or the RiAS-oligos of claim 11 as an effective ingredient.

23. The pharmaceutical composition according to claim 22, which is used for treatment cancer, immune diseases, infectious diseases, or other human diseases caused by aberrant gene expression.

FIG. 1

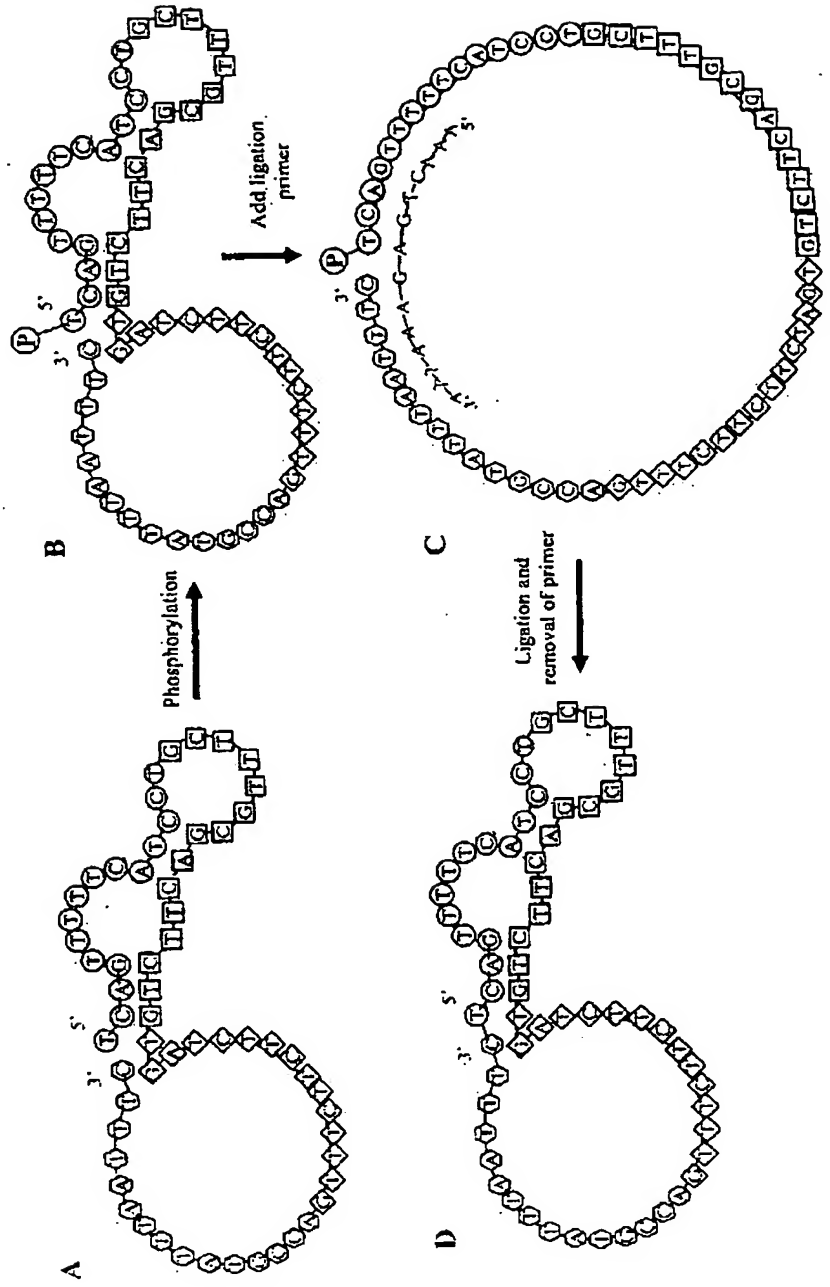

Add ligation primer
Phosphorylation
Ligation and removal of primer
A
B
C
D

FIG. 2

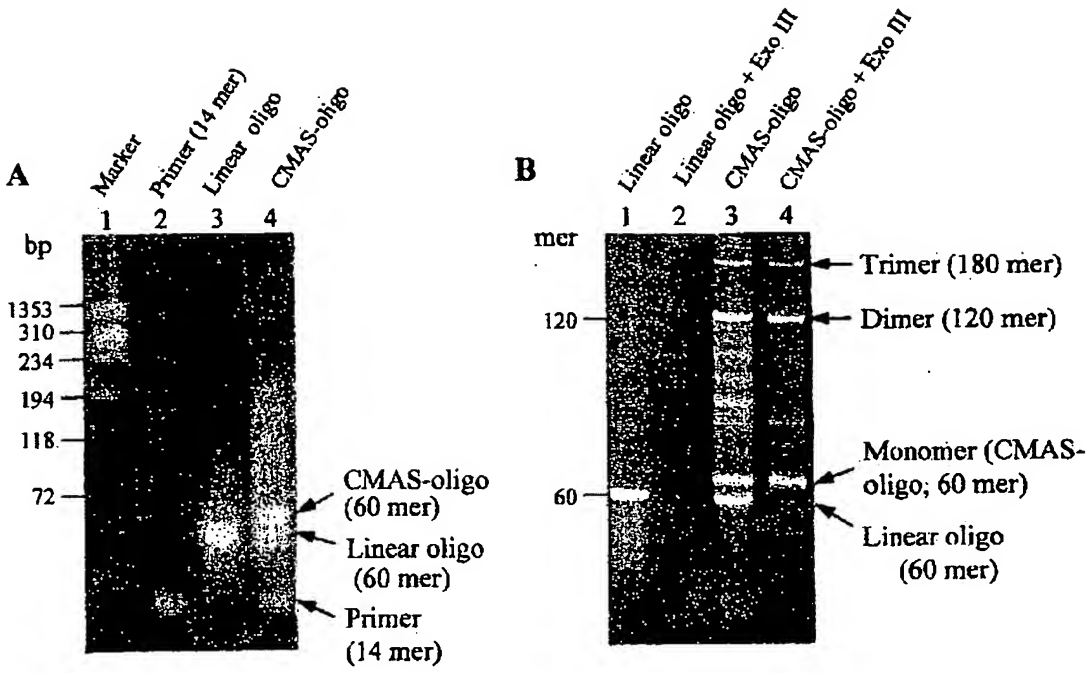

A
Marker
Primer (14 mer)
Linear oligo
CMAS-oligo
1 2 3 4
bp
1353
310
234
194
118
72
CMAS-oligo (60 mer)
Linear oligo (60 mer)
Primer (14 mer)
B
Linear oligo
Linear oligo + Exo III
CMAS-oligo
CMAS-oligo + Exo III
1 2 3 4
mer
120
60
Trimer (180 mer)
Dimer (120 mer)
Monomer (CMAS-oligo; 60 mer)
Linear oligo (60 mer)

FIG. 3

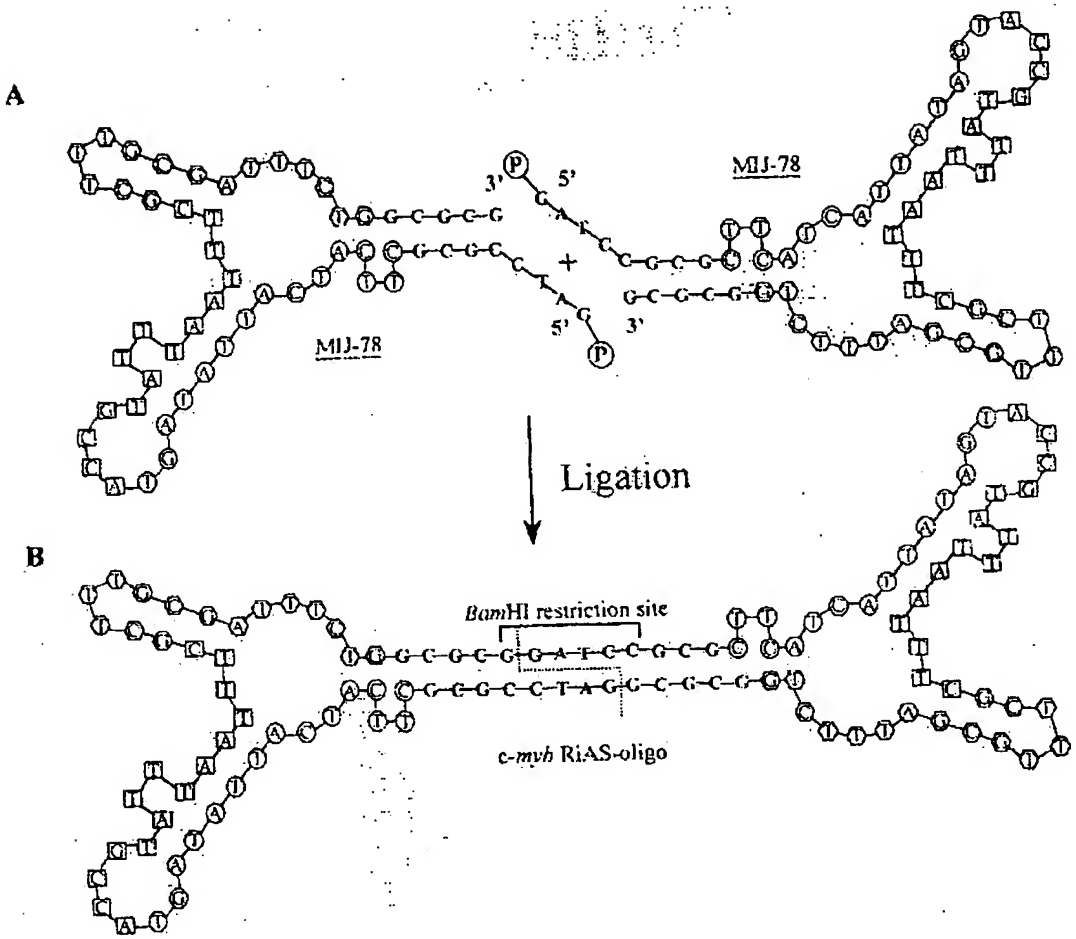

A
MIJ-78
3'
5'
5'
3'
MIJ-78
Ligation
B
BamHI restriction site
c-myb RiAS-oligo

FIG. 4

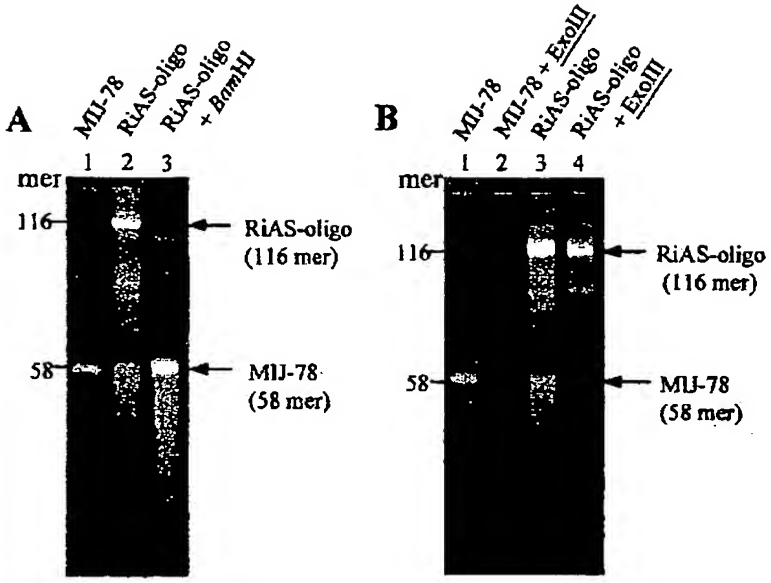

A
MIJ-78
RiAS-oligo
RiAS-oligo + BamHI
1 2 3
mer
116
58
RiAS-oligo (116 mer)
MIJ-78 (58 mer)
B
MIJ-78
MIJ-78 + ExoIII
RiAS-oligo
RiAS-oligo + ExoIII
1 2 3 4
mer
116
58
RiAS-oligo (116 mer)
MIJ-78 (58 mer)

FIG. 5

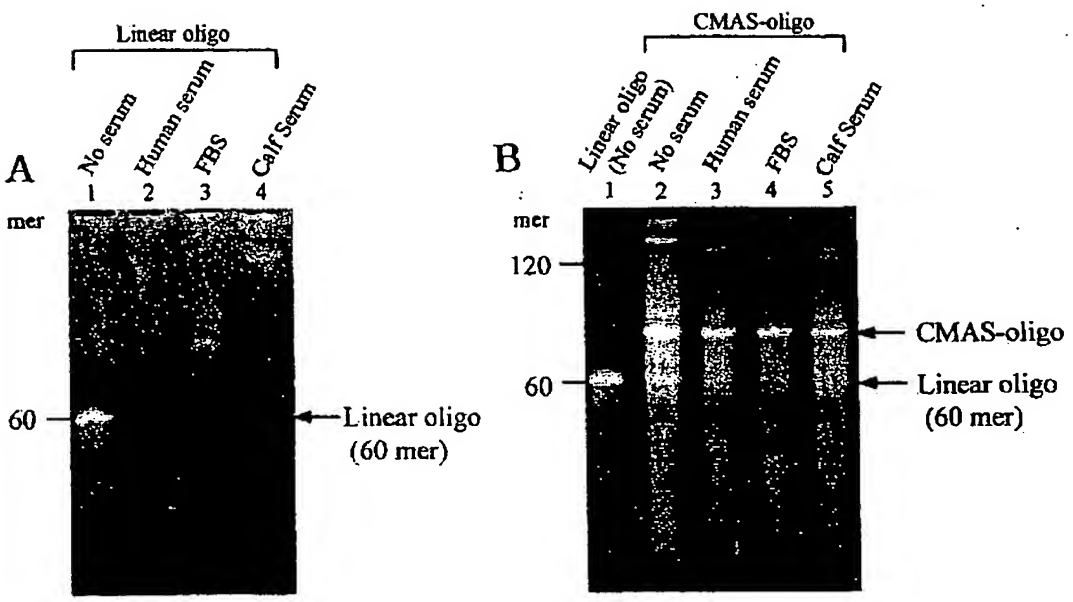

A
Linear oligo
No serum
Human serum
FBS
Calf Serum
1
2
3
4
mer
60
Linear oligo
(60 mer)
B
CMAS-oligo
Linear oligo
(No serum)
No serum
Human serum
FBS
Calf Serum
1
2
3
4
5
mer
120
60
CMAS-oligo
Linear oligo
(60 mer)

FIG. 6

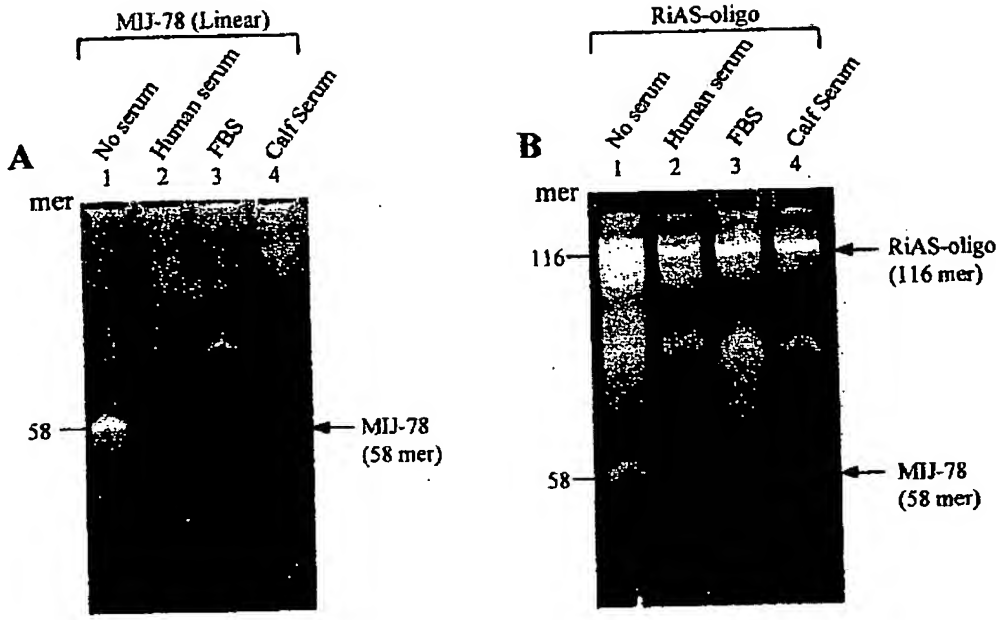

A
MIJ-78 (Linear)
No serum
Human serum
FBS
Calf Serum
1
2
3
4
mer
58
MIJ-78
(58 mer)
B
RiAS-oligo
No serum
Human serum
FBS
Calf Serum
1
2
3
4
mer
116
58
RiAS-oligo
(116 mer)
MIJ-78
(58 mer)

FIG. 7

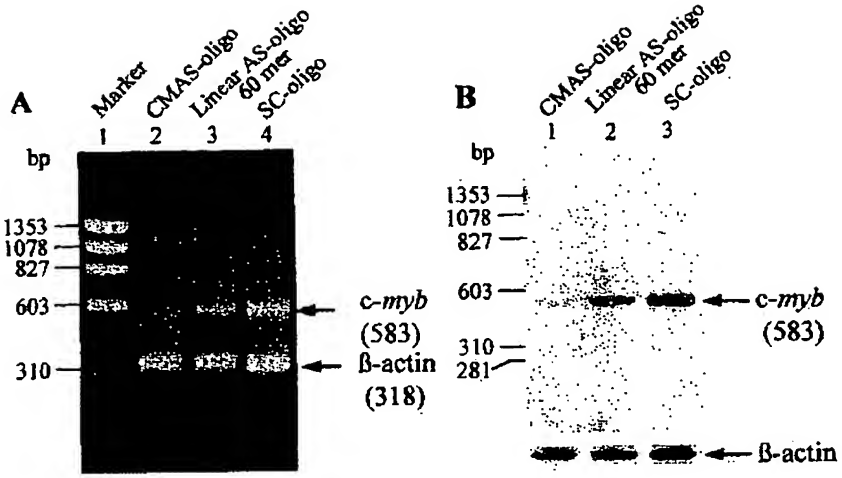

A
Marker
CMAS-oligo
Linear AS-oligo 60 mer
SC-oligo
1 2 3 4
bp
1353
1078
827
603
310
c-myb (583)
ß-actin (318)
B
CMAS-oligo
Linear AS-oligo 60 mer
SC-oligo
1 2 3
bp
1353
1078
827
603
310
281
c-myb (583)
ß-actin

FIG. 8

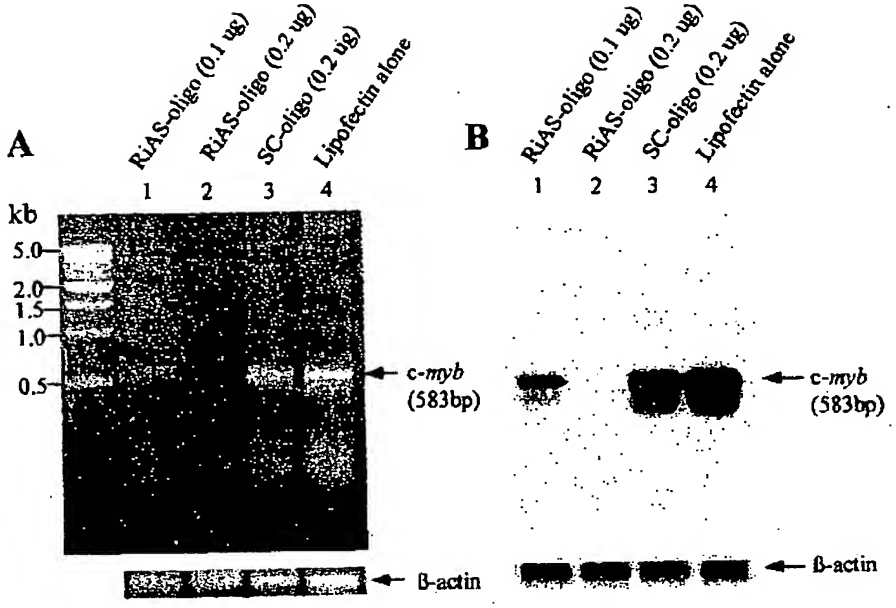

A
RiAS-oligo (0.1 ug)
RiAS-oligo (0.2 ug)
SC-oligo (0.2 ug)
Lipofectin alone
1 2 3 4
kb
5.0
2.0
1.5
1.0
0.5
c-myb (583bp)
ß-actin
B
RiAS-oligo (0.1 ug)
RiAS-oligo (0.2 ug)
SC-oligo (0.2 ug)
Lipofectin alone
1 2 3 4
c-myb (583bp)
ß-actin

FIG. 9

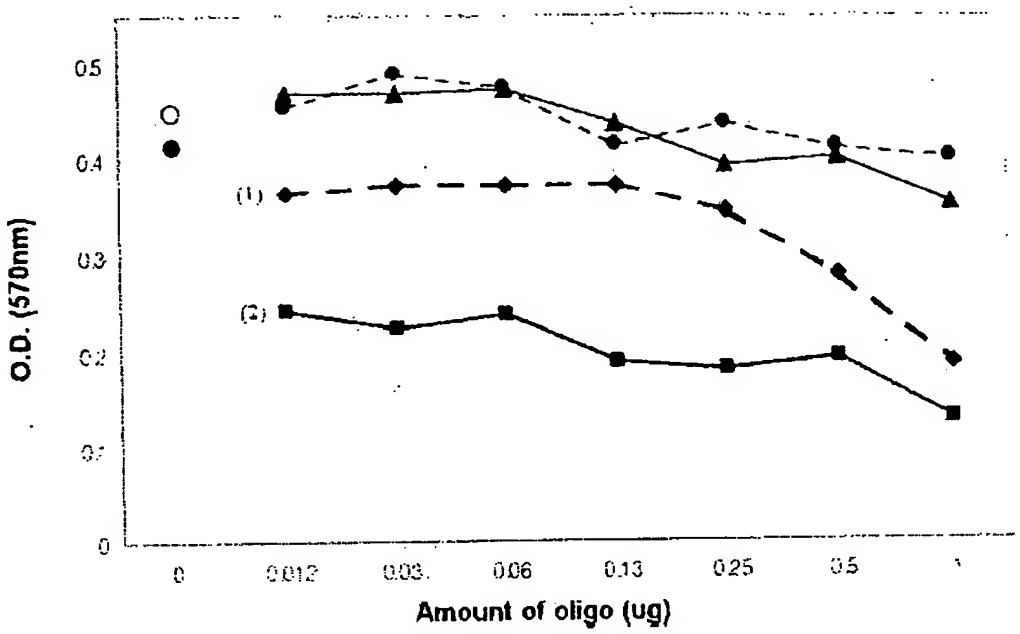

O.D. (570nm)
0.5
0.4
0.3
0.2
0
(1)
(2)
0
0.012
0.03
0.06
0.13
0.25
0.5
Amount of oligo (ug)

FIG. 10

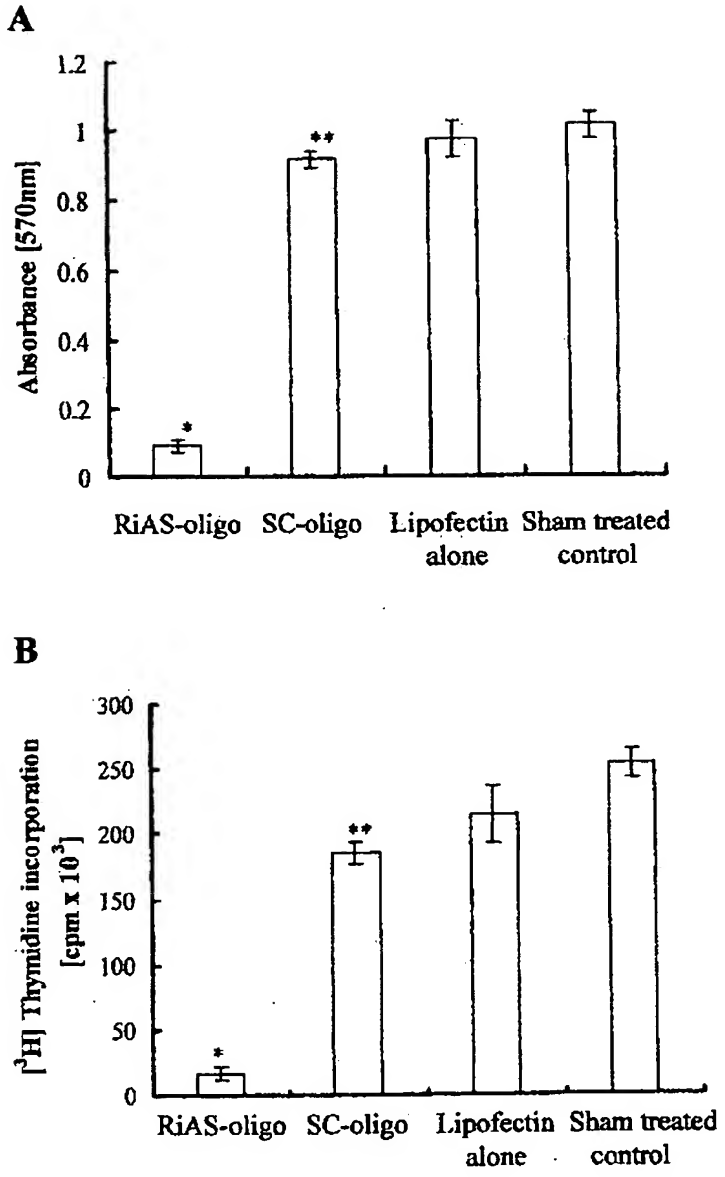

A
Absorbance [570nm]
1.2
1
0.8
0.6
0.4
0.2
0
*
**
RiAS-oligo
SC-oligo
Lipofectin alone
Sham treated control
B
[3H] Thymidine incorporation [cpm x 10^3]
300
250
200
150
100
50
0
*
**
RiAS-oligo
SC-oligo
Lipofectin alone
Sham treated control

FIG. 11

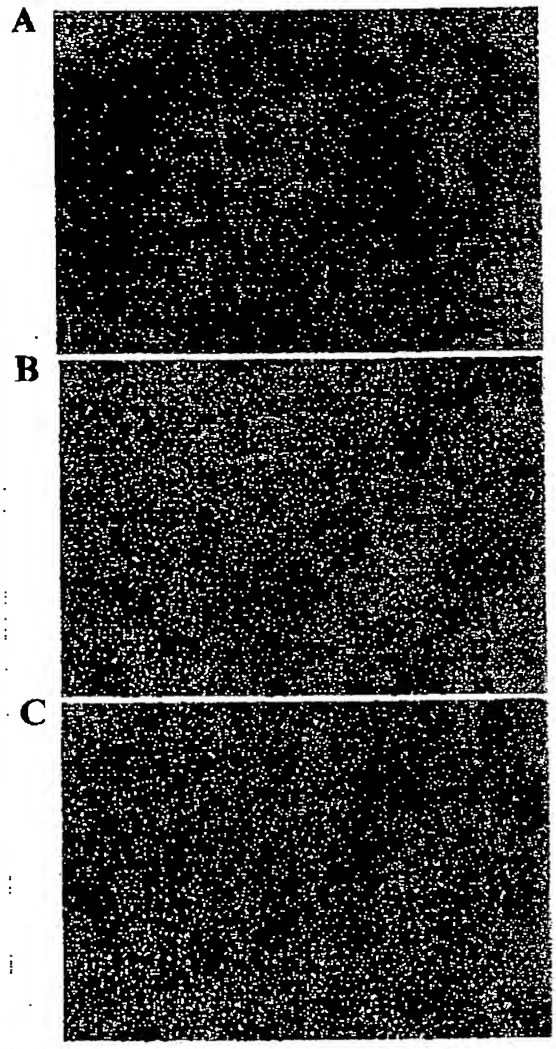


FIG. 12

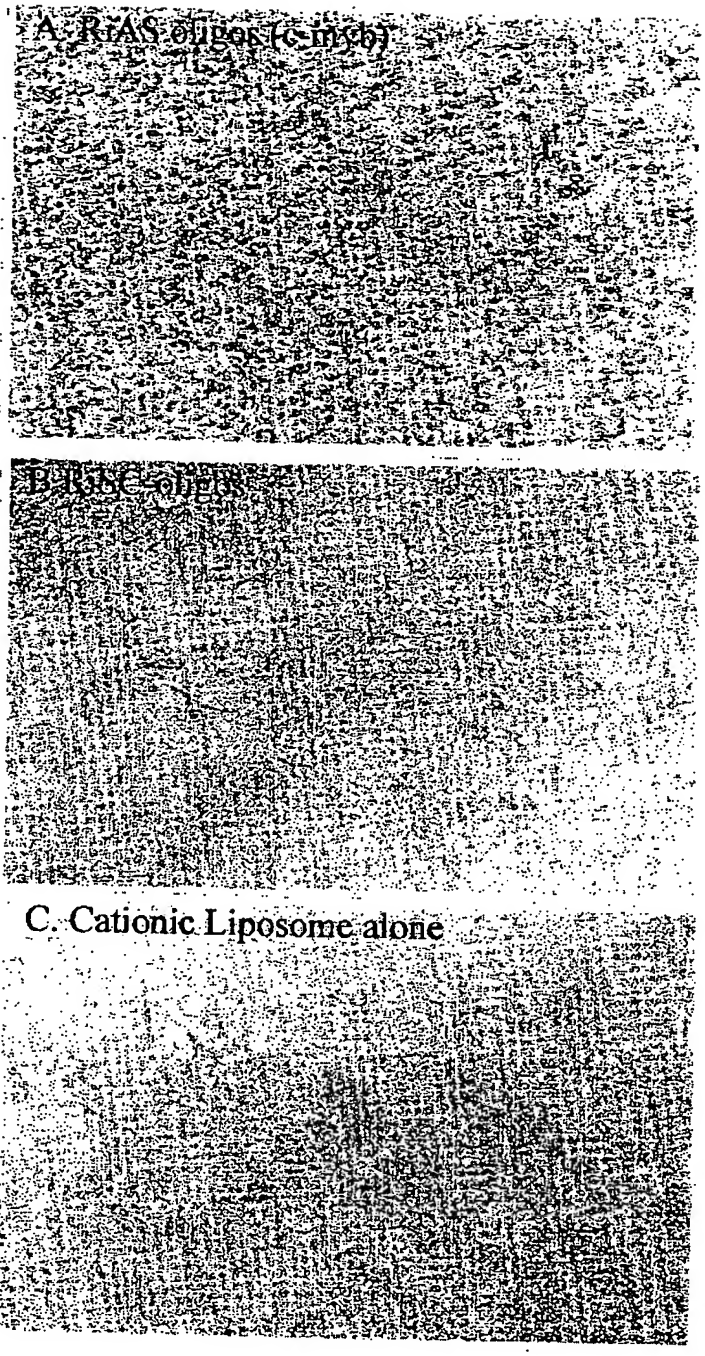


FIG. 13

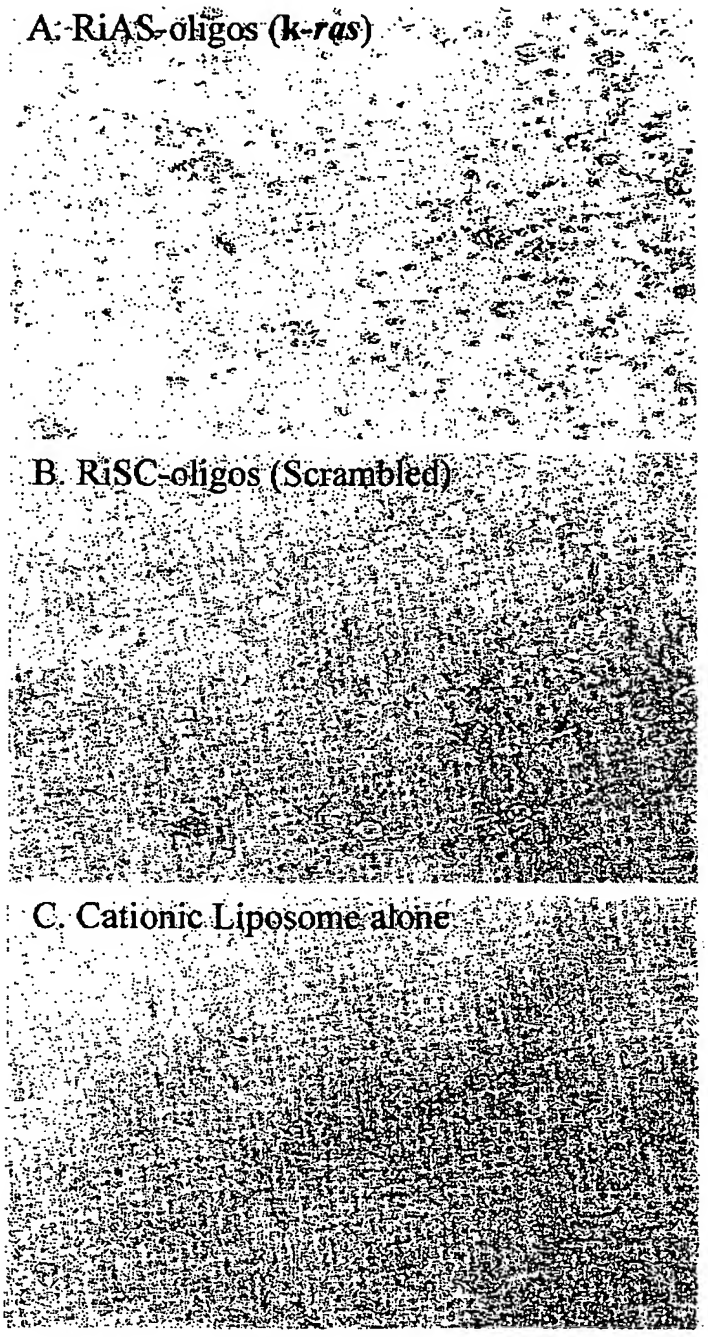
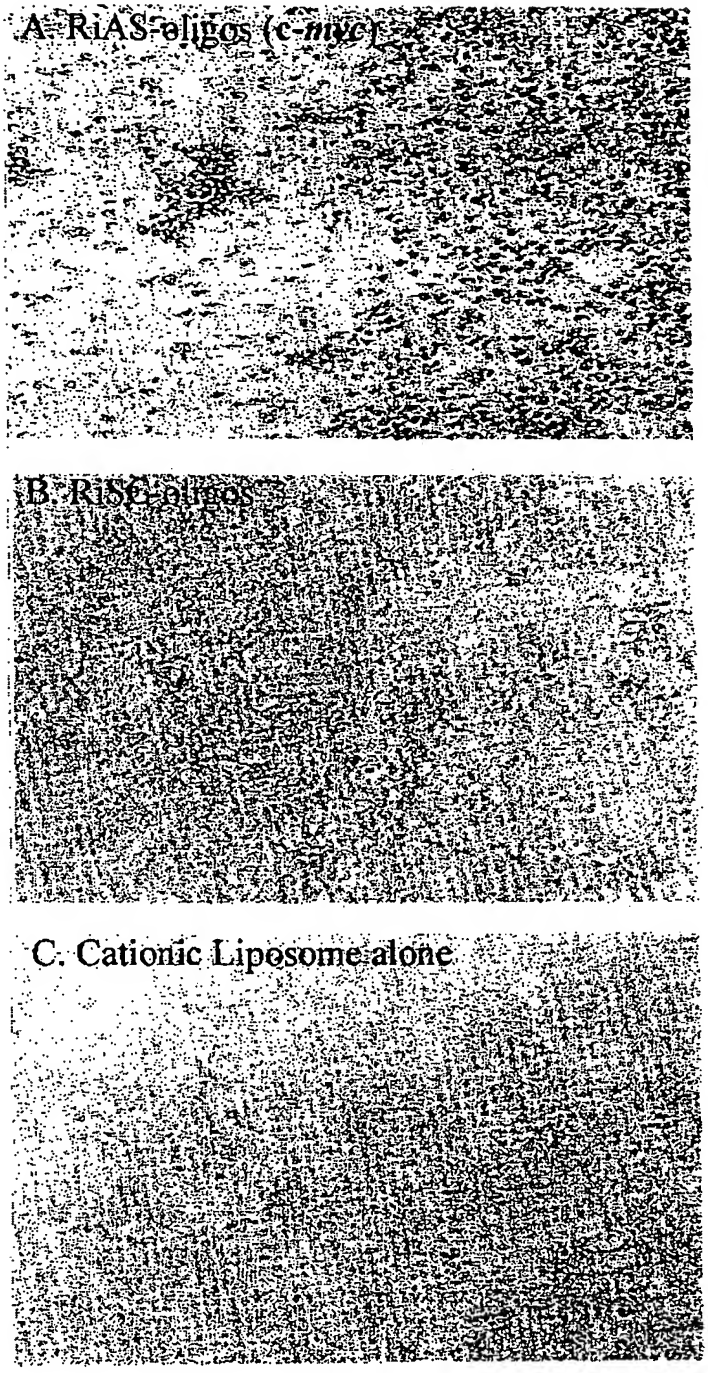

A. RiAS-oligos (k-ras)
B. RiSC-oligos (Scrambled)
C. Cationic Liposome alone

FIG. 14



SEQUENCE LISTING

<110> PARK, Jong-Gu

<120> The novel antisense oligos with better stability and antisense effect

<130> 0fpo-02-10

<150> KR 99-122917
<151> 1999-04-08

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<400> 4
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<400> 5
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<211> 60
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<213> Artificial Sequence

<220>
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60

<210> 7
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> antisense oligonucleotides

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<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> internal primer

<400> 8
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<210> 9
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<213> Homo sapiens

<400> 9
tattcttctg ctcta 15

<210> 10
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<212> DNA
<213> Homo sapiens

<400> 10
cccagtctct tgtgtgc 17

<210> 11
<211> 18
<212> DNA
<213> Homo sapiens

<400> 11
tggcgcggcg ggcggcgg 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00305

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07H 21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

C07H 21/00

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the international search (name of data base and, where practicable, search trerms used)

NCBI, pubmed , IBM patent database, USPTO patent database "antisense",

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Biol Chem Feb 2000, Vol 275, No 7, pages 4747-53	1-23
X	Biochem J Mar 2000, vol 346, Pt2, pages 295-303	1-23
A	US 5985620 A(Gene Shears Pty.Ltd) 16 Nov. 1999(16. 11. 1999) column 1,2 column 8,9,10	1-19
A	US 5939262 A(Gene Shears Pty.Ltd) 16 Nov. 1999(16. 11. 1999) column 5,6,7	1-19

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 JULY 2000 (31.07.2000)	04 AUGUST 2000 (04.08.2000)
Name and mailing address of the ISA/KR Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LIM, Hea Joon Telephone No. 82-42-481-5590

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